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# Induction of Mammary Tumors by Expression of Polyomavirus Middle T Oncogene: A Transgenic Mouse Model for Metastatic Disease

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**The effect of mammary gland-specific expression of the polyomavirus middle T antigen was examined by establishing lines of transgenic mice that carry the middle T oncogene under the transcriptional control of the mouse mammary tumor virus promoter/enhancer. By contrast to most transgenic strains carrying activated oncogenes, expression of polyomavirus middle T antigen resulted in the widespread transformation of the mammary epithelium and the rapid production of multifocal mammary adenocarcinomas. Interestingly, the majority of the tumor-bearing transgenic mice developed secondary metastatic tumors in the lung. Taken together, these results suggest that middle T antigen acts as a potent oncogene in the mammary epithelium and that cells that express it possess an enhanced metastatic potential.**

The molecular basis underlying the ability of tumor cells to metastasize from the primary site of growth to other tissues is a major challenge in understanding oncogenesis. Metastasis likely involves a complex interaction between tumor cells, the extracellular matrix, adjacent stromal cells, and blood and/or lymphatic vessels. The products of several genes have been implicated as important determinants of the metastatic potential of a tumor cell. These include various proteases that are thought to play important roles in the turnover of basement membrane components such as collagen, glycoproteins, and proteoglycans. Evidence from a number of studies has suggested that the balance between expression of proteolytic enzymes and their inhibitors plays an important role in tumor invasion (22). In addition to the activation of proteases, metastasis may also involve alteration of cell surface determinants such as CD44 (14) or the function of the intracellular protein NM23 (20). Although the products of these genes contribute to the overall metastatic phenotype, the underlying causes responsible for their deregulation are still poorly understood. Ultimately, metastasis is thought to result from the aberrant expression of oncogenes or inhibition of their cognate regulators. For example, amplification and overexpression of a variety of oncogene products such as Neu/ErbB-2 and Int-2 have been inversely correlated with relapse and survival of affected cancer patients (21, 34).

While these clinical studies have provided important insights into oncogenesis, another useful model that has been used to study the role of oncogenes in tumor progression is the transgenic mouse. Transgenic mouse strains that express activated oncogenes in a variety of tissue types have been generated by a number of laboratories (16). Although many of these strains develop heritable malignancies, both the kinetics and apparent clonal nature of these tumors argue that additional genetic events are required for the cell to acquire the full malignant phenotype (16). By contrast to these observations, animals of one strain of transgenic mice uniformly expressing the activated *neu* tyrosine kinase under the transcriptional control of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) develop adenocarcinomas involving the entire mammary epithelium (26). Because these tumors arise synchronously and are polyclonal in origin, it was concluded that the expression of the activated *neu* protein was sufficient for transformation of the primary epithelial cell. These observations suggest that expression of activated *neu* tyrosine kinase at a certain threshold in the mammary epithelium can obviate the requirement for additional genetic alterations.

Another potent tyrosine kinase activity that has been implicated in the genesis of murine mammary tumors is that associated with the polyomavirus (PyV) middle T antigen. Infection of newborn or *nu/nu* mice with PyV results in the formation of a number of epithelial and mesenchymal tumor types of which mammary adenocarcinomas represent a significant proportion (4, 12, 37). Genetic analyses of PyV-mediated tumorigenesis has shown that a functional middle T antigen is required for tumor induction (17). The potent transforming activity of middle T antigen is dependent on its association with a number of cellular proteins. For example, there is compelling evidence that middle T specifically associates with and activates the tyrosine kinase activity of a number of c-*src* family members (c-*src*, c-*yes*, and *fyn*) (5, 7, 11, 18, 19). Furthermore, formation of this complex appears to be critical for middle T antigen to transform cells (9). In addition to association with tyrosine kinases, middle T antigen is also known to interact with the 85-kDa subunit of the phosphatidylinositol 3′-kinase (10, 40), and this association is also required for its transforming activity (37). More recently, stable complexes between protein phosphatase subunits A (regulatory) and C (catalytic) and middle T antigen have also been detected (27, 39). However, the role of such complexes in oncogenesis is unknown.

Given the ability of PyV middle T antigen to affect signal cell proliferation through a number of signal transduction pathways, we assessed its oncogenic potential in the mammary gland. To accomplish this, we directed the expression of the middle T antigen to the mammary epithelium by

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isolating transgenic mice carrying an MMTV-PyV middle T antigen fusion gene. Expression of middle T antigen in several independent transgenic strains resulted in the synchronous appearance of multifocal tumors involving all mammary glands. Because these tumors occurred early in mammary gland development and affected all transgenic animals, expression of the middle T oncogene appears to result in rapid conversion of the mammary epithelium to the transformed state. Interestingly, a majority of the middle T transgenic mice developed multiple metastases in the lung. The multifocal nature of these tumors and the high incidence of metastatic disease observed in these strains have important implications for understanding the molecular basis of tumor progression.

## MATERIALS AND METHODS

**DNA constructions.** To derive the pMMTV MT construct, plasmid pmT165 (9) bearing the cDNA encoding the PyV middle T antigen (bounded by nucleotides 154 to 1560) (38) was cleaved with *Hin*dIII and *Eco*RI and inserted into corresponding *Hin*dIII and *Eco*RI sites of the PA-9-derived expression vector, pMMTV-SV40. The latter construct was established by first inserting the *Pst*I-to-*Bam*HI fragment bearing the simian virus 40 (SV40) small t splicing and polyadenylation signal from CDM8 (32) into the corresponding sites in plasmid Bluescript KS (Stratagene) and then cloning the MMTV LTR containing *Sal*I-to-*Hin*dIII fragment derived from plasmid pMMTV neuNT (26) into the corresponding sites of Bluescript KS. The β-casein riboprotection probe was obtained from J. Rosen and was cloned as a 205-bp *Pst*I fragment in plasmid pSP64 (Promega). The PyV middle T riboprotection probe pSP65mT (HA) was obtained from J. Hassell and contains a 203-bp *Hin*dIII-to-*Acc*I fragment of the PyV early region (PyV nucleotides 165 to 368) (35) inserted into the *Hin*dIII and *Acc*I sites of pSP65 (Promega). Finally, the internal control plasmid rpL 32 27.3.7, obtained from M. Shen, encodes an *Xho*II-to-*Dra*I fragment of the mouse ribosomal gene L32 inserted into the corresponding sites of plasmid Bluescript KS. All plasmids were isolated as described previously (33).

**Generation and identification of transgenic mice.** DNA was prepared for microinjection by digestion with 4 U each of *Sal*I and *Spe*I per μg for 1.5 h. The DNA was electrophoresed through a 1% agarose gel and purified as described previously (33). FVB female mice (Taconic Farms, Germantown, Pa.) were mated with FVB males the night before injection. After isolation of the fertilized one-cell mouse embryos, the pronuclei of these zygotes were injected with 0.5 to 1 pl of DNA solution (5 μg/ml). Following microinjection, viable eggs were washed once in M2 medium (29a) and transferred to the oviducts of pseudopregnant Swiss-Webster mice (Taconic Farms). To identify transgenic progeny, genomic DNA was extracted from a 1.5-cm tail clipping as described by Muller et al. (26). The nucleic acid pellet was resuspended in 100 μl of distilled water at an approximate DNA concentration of 1 μg/ml, and 15 μl of the DNA solution was digested with 30 U of *Bam*HI for 1.5 h. After gel electrophoresis and Southern blot transfer (36), the GeneScreen filters (Dupont) were hybridized with a PyV middle T cDNA probe radiolabelled with [α-$^{32}$P]dCTP by random priming (13).

**Expression data.** RNA was isolated from tissues by the procedure of Chirgwin et al. (8), using the CsCl sedimentation gradient modification. RNA yield was determined by UV adsorption at 260 nm after dissolving in sterile $H_2O$. RNA probes were made with either the Bluescript (Stratagene Inc., San Diego, Calif.) or pGEM vectors, and RNase protection assays were performed as described by Melton et al. (23), using 10 μg of total cellular RNA per assay.

**In vitro kinase analyses.** In vitro kinase assays were conducted as described by Aguzzi et al. (2). Tissue samples were ground to a fine powder by using a pestle and mortar cooled with liquid nitrogen and were lysed with 20 mM Tris (pH 8.0)–150 mM NaCl–1% Nonidet P-40–2.5 mM EDTA–1 mM sodium orthovanadate–10 mM NaF–1% aprotinin–10 mM leupeptin; 500 μg of total protein and 1 μg of rat polyclonal 3A1 (24) were incubated for 1 h at 4°C and then incubated with 30 μl of protein A-Sepharose beads for an additional hour. After five washes with TNE (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA), the beads were resuspended in 25 μl of kinase buffer (20 mM morpholinepropanesulfonic acid [pH 7.0], 5 mM $MgCl_2$, 5 μCi of [γ-$^{32}$P]ATP) and incubated at 30°C for 20 min. Following a wash with 1 ml of TNE, the beads were resuspended in 50 μl of sample buffer (10 mM Tris [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol, 5% β-mercaptoethanol) and electrophoresed on 10% SDS-polyacrylamide gels.

**Histological evaluation.** Complete autopsies were performed as described by Muller et al. (26). Tissues were fixed in 4% paraformaldehyde, blocked in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin, and examined as indicated in the legend to Fig. 3.

## RESULTS

**Generation of MMTV-PyV middle T antigen mice and tissue specificity of transgene expression.** To derive transgenic mice expressing PyV middle T antigen in the mammary gland, a cDNA encoding PyV middle T antigen (38) was inserted into an MMTV LTR expression vector (Fig. 1A). The MMTV component was derived from plasmid PA9 (15), whereas the SV40 transcriptional processing signals at the 3′ end of the cDNA were obtained from plasmid CDM8 (32). To ensure that the MMTV/middle T antigen recombinant was biologically active, the transforming potential of the fusion gene was first assessed by transfection into Rat-1 cells. As expected, this construct was capable of transforming Rat-1 cells in the presence of supplemented glucocortocoids (data not shown). Before this plasmid was microinjected into one-cell mouse embryos, plasmid sequences were released by digestion with *Sal*I and *Spe*I (Fig. 1A). After injection of mouse zygotes, seven transgenic founder animals (MT#121, MT#196, MT#235, MT#634, MT#654, MT#668, and MT#670) were generated. With the exception of the two founder animals MT#235 and MT#196, both of which failed to transmit the transgene, the founders passed the transgene to their progeny in a Mendelian fashion.

To assess the tissue specificity of transgene expression, 10 μg of total RNA isolated from a variety of tissues was subjected to RNase protection analyses. As shown in Fig. 1B, the probe yields a 203-base protected fragment corresponding to the 5′ portion of the middle T cDNA. To ensure that approximately equal amounts of RNA from all organs were analyzed, an antisense probe from the mouse ribosomal protein L32-4A (kindly provided by M. Shen) was included in each hybridization reaction as an internal control. Representative results from these RNase protection analyses are shown for the MT#634 line in Fig. 1B. Both male and female carriers derived from this line developed extensive mammary tumors with early onset (Table 1). Female transgenic mice expressed high levels of the trans-

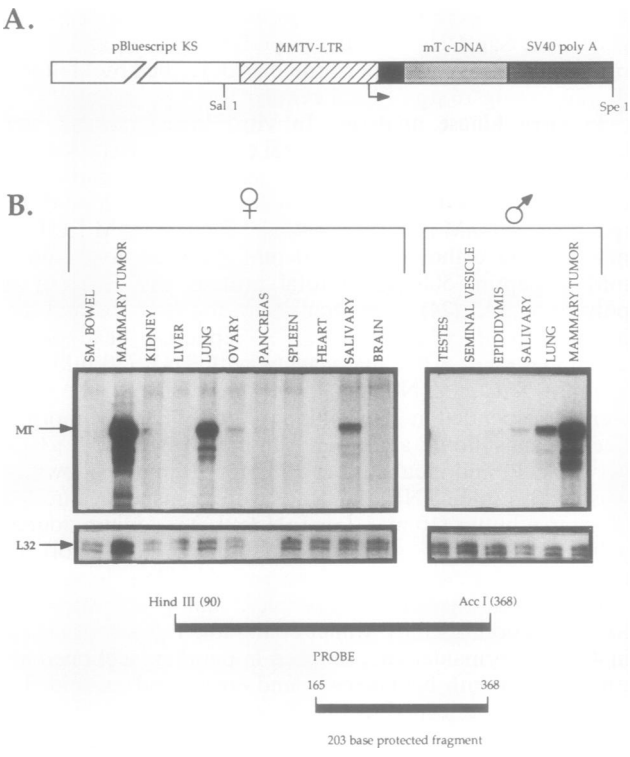


FIG. 1. Structure of transgene and tissue specificity of transgene expression. (A) Transgene structure. The unshaded region represents the sequences within the Bluescript vector backbone, the striped portion contains the MMTV LTR derived from plasmid pA9 (15), the filled region corresponds to an inert region derived from the original pA9 vector, the stippled region contains the cDNA encoding the PyV middle T antigen, and the adjacent cross-hatched region contains the transcriptional processing sequences derived from the SV40 early transcription unit. Relevant restriction sites and transcription start site (indicated by the arrow) are also shown. (B) RNA transcripts corresponding to the MMTV/middle T transgene in various organs of the MT#634 transgenic strain. Tissues were derived from a multiparous MT#634 female at 119 days of age (MT 5268) and an MT#634 male at 119 days of age (MT 2833). The antisense probe used in this RNase protection analysis (shown at the bottom) protects a 203-nucleotide fragment marked by MT and an arrow. Nucleotide numbers refer to the PyV early-region nucleotide sequence (35). Also shown is the RNase protection analysis with an antisense probe directed against the mouse L32 ribosomal gene. The L32 probe protects a 278-nucleotide fragment and is marked by L32 and an arrow. A lower band is also consistently observed in these RNase protections with the L32 probe.

gene product in the mammary tumors, with lower levels detected in the ovaries and salivary glands. Interestingly, in older (2 to 3 months) female transgenic animals, middle T transcripts were also detected in the lungs (Fig. 1B and 4B). This lung-specific expression was not observed in younger animals and is correlated with the appearance of multifocal lung metastases (see Fig. 4A). Male transgene carriers expressed high levels of the fusion gene in mammary tumors and lung metastases, whereas lower levels were detected in the salivary glands and epididymis.

The tissue specificity of transgene expression was also assessed for the remaining six transgenic animals of MMTV/middle T antigen mice by using the same RNase protection probe. As shown in Table 1, variable levels of transgene expression were noted in mammary glands of female transgenic mice derived from the MT#121, MT#668, MT#654, and MT#670 lines. Among the different female transgenic animals, considerable variation in both the amount and the temporal pattern of transgene expression was observed. For example, transgene transcripts were readily detected in the mammary glands derived from virgin female carriers of the MT#634 and MT#668 lines. By contrast, at least two pregnancies were required in order to detect similar levels of transgene expression in the MT#121, MT#654, and MT#670 strains (Table 1). As observed with the other transgenic strains, the appearance of these tumors was strictly correlated with the expression of the transgene. With the possible exception of the MT#196 transgenic founder, which developed mammary tumors, a seminal vesicle neoplasm, and hemanginomas, the lower amounts of middle T antigen RNA observed in the various tissues were not associated with any apparent growth disturbance.

To confirm that these transcripts encoded a functional middle T antigen, tissue extracts derived from the mammary glands of several of these transgenic lines were subjected to in vitro kinase assays using polyclonal antisera directed against middle T antigen. As a consequence of PyV middle T antigen's ability to associate with and activate a number of the *src* tyrosine kinases, the middle T protein becomes autophosphorylated on tyrosine residues in vitro (5, 7, 11, 18, 19). As illustrated in Fig. 2, a prominent 56-kDa phosphorylated band was observed in lanes incubated with the middle T-specific antisera. Because the band observed in the tumor extracts comigrated with middle T antigen derived from a Rat-1 cell line expressing middle T antigen, these observations suggest that the tumor extract possesses middle T-associated kinase activity. Incubation of the extracts with a nonspecific control antibody (mouse immunoglobulin G) resulted in the appearance of a background phosphorylated band that is present in all lanes. Consistent with the results of these in vitro kinase assays, Western immunoblot analyses also showed the presence of 56-kDa middle T protein in these tumors (data not shown). Together, these results indicate that the MMTV/middle T transgene in these strains associates with an active tyrosine kinase in the mammary epithelium.

**Expression of the PyV middle T antigen in the mammary epithelium results in the generation of multifocal mammary tumors.** Elevated expression of middle T antigen in the mammary glands of transgenic mice had dramatic consequences. In three of the five characterized transgenic lines, high levels of transgene expression were initially associated with the inability of female carriers to nurse their young. In addition, the MT#235 founder animal displayed an inability to lactate. In two of these transgenic lines (MT#634 and MT#668), this phenotype was apparent during the initial pregnancy, but the MT#121 strain demonstrated the nursing defect only after multiple pregnancies. Although there was some variation between these strains with respect to appearance of this phenotype, the inability to nurse was closely correlated with the onset of transgene expression (data not shown). By comparison with virgin female normal mammary tissue (Fig. 3A), whole-mount examination of virgin female mammary tissue from the MT#634 strain (3 weeks of age) revealed the presence of multiple mammary adenocarcinomas (Fig. 3B). These tumors were generally highly fibrotic, with dense connective tissue separating individual nests of tumor cells (Fig. 3C). By 5 weeks of age, all female carriers from the MT#634 ($n$ = 35) and MT#668 ($n$ = 4) lines had developed palpable mammary tumors (Table 1) that involved the entire mammary fat pad (Fig. 3C). The multifocal ap-

TABLE 1. Transgene expression and onset of tumors in MMTV/middle T mice[a]

| Line | Sex[b] | Expression in: | | | | | | | Onset of tumor formation (days) | Tumor type(s) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | M.gl. | Sal | L | O | SV | T | Epi | | |
| MT#121 | F | +++ | − | + | + | | | | 94 ± 18 ($n$ = 20) | M.gl. adenocarcinoma |
| | M | − | − | − | | − | − | − | NA | No tumor |
| MT#196 (founder) | M | +++ | + | + | | ++ | ND | ND | 25 ($n$ = 1) | Adenocarcinoma of M.gl. and seminal vesicles, hemangiomas |
| MT#235 (founder) | F | +++ | − | − | + | | | | 70 ($n$ = 1) | M.gl. adenocarcinoma |
| MT#634 | F | +++ | + | ++ | + | | | | 34 ± 6 ($n$ = 35) | M.gl. adenocarcinoma |
| | M | +++ | + | + | | − | + | − | 83 ± 20 ($n$ = 20) | M.gl. adenocarcinoma |
| MT#654 | F | +++ | + | + | − | | | | 175 ± 68 ($n$ = 2) | M.gl. adenocarcinoma |
| | M | − | − | − | | − | − | − | NA | No tumor |
| MT#668 | F | +++ | ++ | +++ | − | | | | 36 ± 2 ($n$ = 3) | M.gl. adenocarcinoma |
| MT#670 | F | +++ | − | + | − | | | | 155 ($n$ = 1) | M.gl. adenocarcinoma |
| | M | − | − | − | | − | − | − | NA | No tumor |

[a] RNase protection analysis was performed on 10 μg of total RNA isolated from a variety of organs in the MMTV/middle T strains as described in Materials and Methods. Relative levels of transgene expression are indicated by + (low), ++ (intermediate), and +++ (high). M.gl., mammary gland; Sal, salivary gland; L, lung; O, ovary; SV, seminal vesicle; T, testes; Epi, epididymis; NA, not applicable; ND, not determined; $n$, number of animals analyzed. All transgenic mice analyzed were tumor bearing.

[b] F, female; M, male.

pearance of mammary tumors in these strains was not dependent on pregnancy, because virgin female carriers displayed an identical tumor phenotype. The appearance of mammary tumors in the MT#121 line was closely correlated with the delayed onset of transgene expression, where 50% of female carriers at risk developed tumors by 94 days (Table 1). Despite the delayed kinetics of tumor formation, all multiparous MT#121 female carriers developed mammary tumors that eventually involved the entire mammary fat pad.

Male transgenic mice ($n$ = 17) derived from the MT#634 strain also developed mammary adenocarcinomas with 100% penetrance, albeit with delayed onset (Table 1). The appearance of mammary tumors in male transgenic mice is consistent with results obtained with both male MMTV/v-Ha-*ras* and MMTV/activated c-*neu* transgenic mice (26, 33) and may result from expression of the oncogene in the male mammary epithelium prior to its normal regression. By contrast, male transgenic mice derived from the MT#121 strain did not develop mammary tumors, perhaps because of delayed onset of transgene expression. Both the rapid kinetics and the global nature of the tumor phenotype exhibited by these MMTV/middle T transgenic mouse strains suggest that expression of middle T antigen at appropriate levels can lead to transformation of the mammary epithelium.

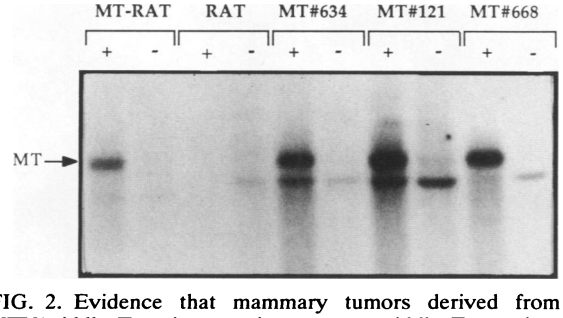


FIG. 2. Evidence that mammary tumors derived from the MMTV/middle T antigen strains possess middle T-associated tyrosine kinase activity. Shown are in vitro kinase activities of mammary tumor extracts derived from multiparous female MT#634 (MT 5524, 76 days old), MT#121 (MT 765, 154 days old), and MT#668 (MT 5532, 85 days old) carriers incubated with polyclonal rat antiserum directed against middle T antigen (+) or nonspecific antibody (−). Also included are a negative control with Rat-1 (RAT) fibroblasts and a positive control with middle T-transformed Rat-1 (MT-RAT) fibroblasts. The 56-kDa phosphorylated middle T antigen is indicated at the left (MT).

**The middle T oncogene induces metastatic disease.** As shown in Table 1, transgene expression was noted in the lung tissue of older individuals derived from the MT#634, MT#668, MT#654, MT#670, and MT#121 lines. Histological examination of lung tissue derived from MT#634, MT#668, MT#654, and MT#121 transgenic mice revealed the presence of multiple foci of metastatic mammary adenocarcinomas lodged in the lung parenchyma (Fig. 3D and 4A). By contrast to the primary mammary tumors, the pulmonary metastases contained little or no connective tissue separating nests of tumor cells (compare Fig. 3C and 3D). Because lung tissue was not obtained from MT#235 and MT#196 founder animals, it was not possible to assess whether middle T antigen expression observed in the lung was the result of metastatic disease. The extent of metastatic involvement in these lines was particularly remarkable with respect to both its degree and penetrance (Fig. 3D and 4A). For example, in the MT#634 strain, 94% of tumor-bearing females developed metastatic disease by 3 months of age. Male MT#634 tumor-bearing animals also developed metastatic disease, albeit with lower penetrance (80%). Similar proportions of the MT#121 (90%) and MT#668 (100%) tumor-bearing animals also developed metastatic disease during a 3-month observation period. Consistent with these observations, metastatic foci could be detected in either the lymphatic or the lung tissue after transplantation of the primary tumors from the tumor-bearing MMTV/middle T transgenic animals into the fat pads of normal syngeneic recipients (data not shown).

While these histological observations strongly suggested that the tumors in the lung were of mammary origin, further molecular analyses with mammary gland-specific probes were performed to establish this point. The metastatic nature of these lung tumors was confirmed by assessing whether these tumors were capable of expressing mammary differentiation markers such as β-casein. Using a probe directed to the 5′ end of the milk gene β-casein, RNase protection

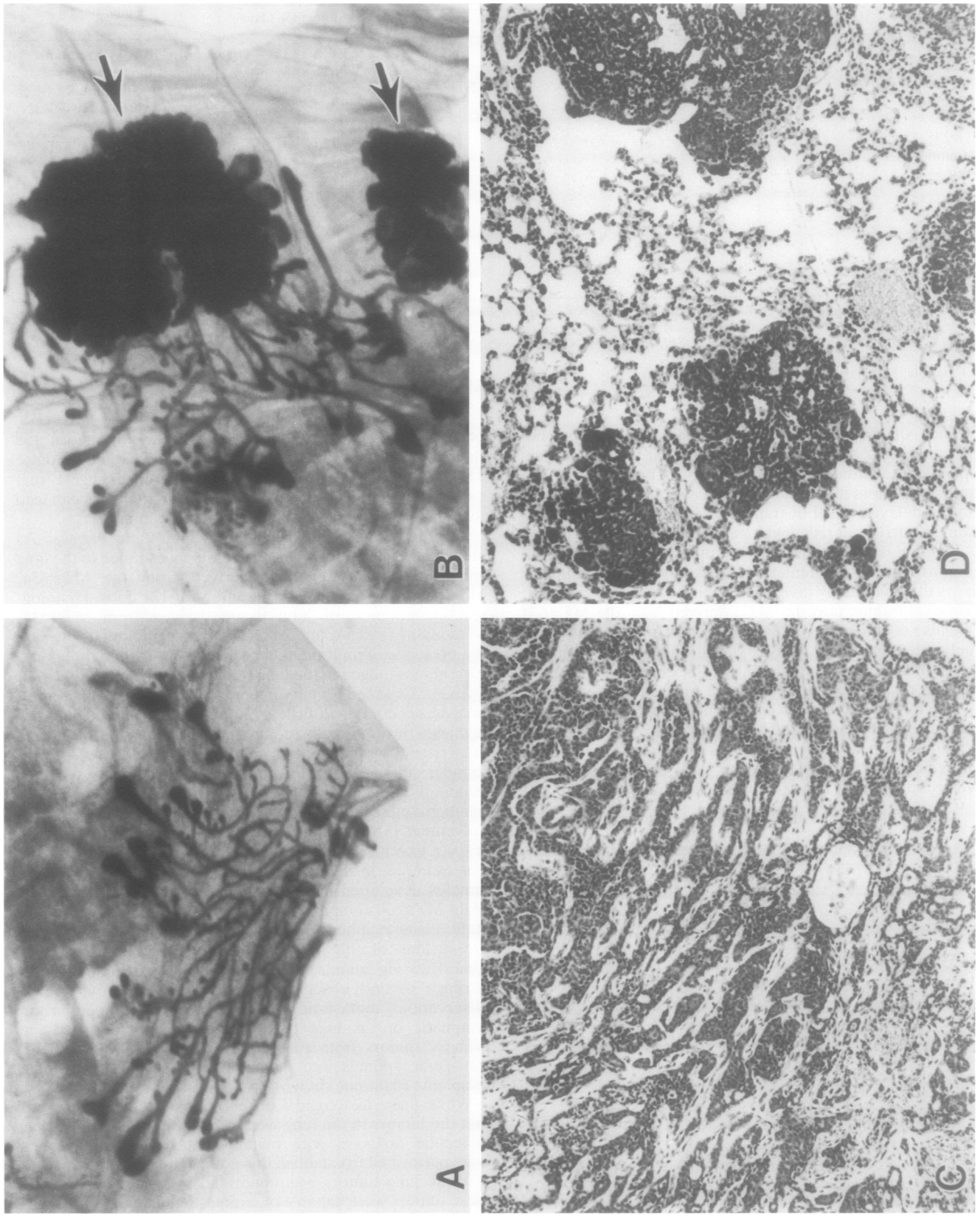
B
D
A
C

FIG. 3. Histopathology of MMTV/middle T transgenic mice. (A) Photomicrograph of a hematoxylin-stained whole mount of the mammary fat pad of a wild-type virgin mouse at 3 weeks of age showing normal growth and development. Magnification, ×16. (B) Photomicrograph of a hematoxylin-stained whole mount of the mammary fat pad of an MT#634 virgin transgenic female (MT 907) at 21 days of age. Compare with panel A. Note the irregular formation of side branches, enlarged terminal buds, and two large multilobular tumor masses (arrows). Magnification, ×16. (C) Photomicrograph of a sclerosing mammary adenocarcinoma from a middle T transgenic multiparous female mouse (MT#634 at 110 days). Note the dense connective tissue separating the attenuated cords of poorly differentiated mammary tumor cells. This pattern is typical of these transgenic mice. Magnification, ×87. (D) Photomicrograph of the lungs of the same mouse showing multiple metastases. Note that the tumor cells form well-defined acinar structures, with very little stroma separating the epithelium. Also note that the tumor cells are intra-alveolar rather than intravascular, indicating growth outside of the vessels. Magnification, ×87.

experiments were conducted on total RNA derived from both primary and lung tumors (Fig. 4B). Both the primary mammary tumor and lung metastases from the MT#634, MT#668, and MT#121 lines expressed moderate levels of β-casein transcripts. By contrast, RNA derived from normal lung tissue was completely devoid of any detectable β-casein mRNA. Taken together with the histological observations, these results demonstrate that expression of middle T antigen in the mammary epithelium leads to the development of metastatic disease.

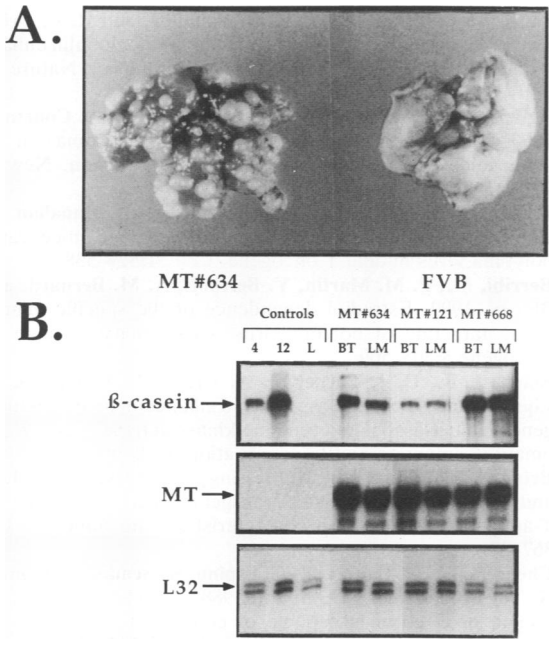


FIG. 4. Evidence that expression of the middle T oncogene results in metastatic mammary adenocarcinomas. (A) Lung tissue isolated from both MT#634 and FVB control animals. Note the extensive metastatic mammary tumors located throughout the lung tissue of the multiparous MT#634 female carrier (MT 5579) at 122 days of age. (B) RNase protection with control and transgenic tissues with probes directed to β-casein, middle T, and the L32 ribosomal internal control. The control tissues were isolated from the mammary glands of normal FVB mice and of 4-day (lane 4)- and 12-day (lane 12)-pregnant mice, as well as from normal lung tissue (lane L). Transgenic tissues derived from multiparous female MT#634 (MT 5579, 122 days), MT#121 (MT 5183, 130 days), and MT#668 (MT 5532, 85 days) carriers include primary breast tumors (lanes BT) and corresponding lung metastases (lanes LM). The 205-nucleotide protected fragment for β-casein, the 203-nucleotide protected fragment for middle T transcript (MT), and the 278-nucleotide protected fragment for the L32 ribosomal control are indicated by arrows.

## DISCUSSION

Analysis of the transforming properties of the PyV middle T oncogene in the mammary epithelium provides important insight into the process of malignant progression. In four independent strains of MMTV/middle T transgenic mice, expression of the transgene ultimately resulted in the uniform morphological transformation of the mammary epithelium. Virgin female transgenic mice derived from the MT#634, MT#235 founder, and MT#668 strains developed multifocal adenocarcinomas as early as 3 weeks of age (Fig. 3B). Both the simultaneous occurrence of these tumors and their multifocal nature suggest that the expression of middle T oncogene is sufficient for mammary epithelial cell transformation.

The potent oncogenic potential of middle T antigen in the mammary gland is further supported by the results obtained with the MT#121 transgenic strain. In this particular transgenic line, mammary gland-specific expression of the middle T transgene was not detected until several pregnancies had occurred (data not shown). However, once transgene expression was observed, these animals developed multifocal mammary adenocarcinomas that eventually involved the entire mammary fat pad. Conceivably, the difference in the kinetics of transgene expression among the various transgenic strains could be influenced by the site of integration of the transgene. For example, variations in both the spatial and temporal patterns of transgene expression were also observed in transgenic mice bearing either the MMTV/activated c-*neu* transgene (26) or an elastase promoter-activated *ras* fusion gene (29). Moreover, the short latency between transgene expression and widespread morphological transformation of the mammary epithelium further argues that progression from a normal epithelial cell to a tumor cell in these mice requires few, if any, additional genetic events.

Consistent with this conclusion, previous studies of PyV middle T antigen in transgenic and chimeric mice have shown similar rapid tumor kinetics. For example, expression of the middle T oncogene under its own promoter or the Moloney murine leukemia virus promoter in transgenic mice results in disseminated endothelial tumors (3, 41). In the latter case, these hemaginomas resulted in embryonic lethality due to early onset of expression of middle T antigen. Because these endothelial tumors were polyclonal in nature and appeared coincident with the first appearance of yolk sac endothelial cells, it was proposed that middle T antigen acted as a single-step oncogene (41). However, because these tumors could potentially recruit normal endothelial cells to the hemaginoma, it was not clear whether all constituent cells were morphologically transformed (42). In another set of experiments, transgenic mice expressing the middle T oncogene in neuronal or epithelial tissues resulted in the formation of multiple neuroblastomas or carcinomas (2, 30).

However, because these transgenic animals exhibited preneoplastic lesions prior to the onset of tumor formation, additional genetic events were likely required.

The rapid tumor progression observed in the middle T oncogene transgenic mice contrasts with the observations made by a number of laboratories with transgenic mice bearing activated oncogenes. For example, multiple genetic events appear to be required for malignant progression in transgenic mice expressing oncogenes such as the SV40 large T antigen gene, c-*myc*, v-Ha-*ras*, or c-*fos* in a variety of different tissue types (1, 6, 31, 33). However, it has recently been reported that animals of one transgenic strain of mice carrying the activated *neu* gene under the transcriptional control of the MMTV LTR develop polyclonal mammary tumors without the need for a second event (26). It is interesting that both the activated *neu* and PyV middle T oncogenes are associated with constitutive tyrosine kinase activities that are refractory to normal cellular regulation. Determination of whether the powerful tissue-specific transforming activity exhibited by these oncogenes reflects the sensitivity of the mammary epithelial cell to a common tyrosine kinase signal transduction pathway awaits further analyses.

While the molecular basis for the potent transforming activity exhibited by the middle T oncogene is unclear, it is conceivable that deregulations of multiple signal transduction pathways through its association with the *src* family of tyrosine kinases, the phosphatidylinositol 3′-kinase, and phosphatase 2A individually contribute to the overall transformed phenotype. Indeed, PyV middle T antigen molecules impaired in their ability to deregulate either of these pathways display a pronounced reduction in the ability to transform cells in vitro or to induce tumors in animals (9, 37). Future experiments directed toward activating each of these signal transduction pathways individually in the mammary gland should allow this question to be addressed.

The unexpected finding that expression of the middle T antigen was closely associated with pulmonary metastases may provide important insight into the metastatic progression. By contrast to other MMTV/oncogene-bearing transgenic mice in which metastasis is a relatively rare occurrence (28), nearly all tumor-bearing MMTV/middle T transgenic carriers thus far analyzed developed metastatic disease. It is likely that these metastatic tumors originate from the primary mammary tumors because they still retain the capacity to express mammary markers such as β-casein. Consistent with this conclusion is the observation that transplantation of these primary mammary tumors into the fat pad of syngeneic recipients resulted in metastasis. The metastatic tumors were restricted to the lung and do not appear to seed other tissue sites. These metastatic foci appear to lodge in the vessels and grow by local expansion and invasion. The apparent specificity of these metastases to the lung may simply reflect the ability of the fine capillary beds of the lung to trap tumor emboli that have entered the bloodstream. Alternatively, the process of metastasis in this system may exhibit target specificity, perhaps mediated through the expression of ligand-specific cell adhesion molecules or the presence of a locally produced growth factor. Given the penetrance of metastatic disease observed in these lines, it is conceivable that middle T is activating cellular genes that are involved in metastatic progression. Because the PyV middle T tumors exhibit elevated proteolytic activity (unpublished observations), genes encoding various members of the protease family and their inhibitors may be potential downstream targets of the PyV middle T-associated tyrosine kinase. In fact, endothelial cells expressing PyV middle T antigen express high levels of urokinase plasminogen activator and low levels of its cognate inhibitor (PAI-1) (25). Determination of whether a similar proteolytic imbalance is responsible for the metastatic phenotype observed in the PyV middle T strains awaits further analysis.

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